NITROGEN METABOLISM IN ERYTHROCYTE MATURATION; RESIDUAL NITROGEN FORMATION AND HEMOGLOBIN SYNTHESIS

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in collaboration with F. Schoelzel

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16. Abstract
The residual nitrogen of rabbit reticulocytes increases during incubation in nutrient-free medium; this does not occur with mature erythrocytes. The increase in residual nitrogen is reduced by the addition of glucose and 11 amino-acids while at the same time an increase in the hemoglobin is observed. An optimum effect is achieved with large phosphate concentrations. The residual nitrogen formation is inhibited by 2.4-dinitrophenol. The source of the residual nitrogen is the reticulocyte stroma.

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NITROGEN METABOLISM IN ERYTHROCYTE MATURATION;
RESIDUAL NITROGEN FORMATION AND HEMOGLOBIN SYNTHESIS[1]

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in collaboration with F. Schoelzel[2]

The development of erythrocytes constitutes a considerable process of specialization whose most important feature is the formation of hemoglobin [1]. At the same time as the hemoglobin is formed, a significant loss of enzymatic power is lost, with disappearance of respiration acquiring particular significance [2, 3]. /33*

The present paper describes experiments which demonstrate the significance of residual nitrogen increase during incubation of blood containing reticulocytes and their composition with hemoglobin synthesis and demonstrate that the stroma is the source of RN.

The closely related problem of the natural substrate of reticulocyte respiration will be the subject of a separate paper.

Methods

Blood rich in reticulocytes was obtained from donor animals.[4]. The reticulocyte count was obtained after staining with brilliant cresyl blue by counting 1,000 cells.

The serum was centrifuged from the cells at 1,200 g, the cells thus obtained washed with the volume of serum obtained three times, and suspended in 0.85% sodium chloride solution or phosphate buffer in a ratio of 1:3. 2 ml were pipetted into Warburg vessels from the suspensions and shaken at 38° for the appointed times. 1 ml of the suspensions was mixed with 3 ml of 7% trichloroacetic acid before and after incubation and the precipitate was separated /34

[1] In this report, the following abbreviations will be used: RN = residual nitrogen, 2,4-DNP = 2,4-dinitrophenol.
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*Numbers in the margin indicate foreign pagination.

by centrifuging. In the supernatent solution, the determination of the RN was carried out using a modification of the method of Lubochinsky and Zalta [5, 6].

The phosphate buffer which was used was isotonic (pH 7.8). The composition of the amino acid mixture corresponded to the essential amino acids of a casein hydrolysate and amounted to approximately 900 micrograms of amino acids per batch. The final concentrations used were 250 mg of glucose/100 ml and 10^{-4} molar 2,4-DNP.

Determination of hemoglobin was carried out after addition of 0.02 ml cell suspension to 10.0 ml of a 0.1% sodium carbonate solution, photometrically using a green filter.

To obtain the stroma, the suspensions were washed once with isotonic sodium chloride solution. Then hemolysis was carried out in m/100 KH_2PO_4, centrifuged at 1,800 g and washed twice with m/100 KH_2PO_4. The resultant stroma was a dirty yellow, the supernatent solution being colorless. This method offers values that are reproducible within ±7%.

Determination of stroma-N was performed after ashing, also with a modification of the method of Lubochinsky and Zalta [6].

Results

1. Residual Nitrogen Formation in Erythrocytes

During the incubation of washed blood corpuscles in physiological saline, residual nitrogen increases significantly (see Table 1). In the blood of normal animals, there is only a slight increase in RN, but no direct relationship between the percentage of reticulocytes and respiration and RN increase could /35 not be found. The RN increase proved to be dependent upon aerobic conditions.

While the addition of glucose under aerobic conditions led to a significant RN saving effect in sodium chloride, behavior was reversed when glucose was added to the phosphate buffer. The addition of the glucose produced an increase in the RN accumulation, small but definite. It must be taken into account that the RN increase in the phosphate buffer is much less than the incubation in saline solution, but without the addition. Under anaerobic conditions, the addition of the glucose in either sodium chloride or phosphate buffer led to a nearly regular increase, as compared with the preparation without the addition.

TABLE 1. INFLUENCE OF OXYGEN AND GLUCOSE ON RN FORMATION

Type of Animal	Animal No.	Experiment No.	Reticulocytes, %	Duration of Incubation, hrs.	RN, mg/ml of Cells: Initial Value	Increase per ml of Cells: Aerobic, Without Glucose	Aerobic, With Glucose	Anaerobic, Without Glucose	Anaerobic, With Glucose
Donor Animals	6	23	27	8	0,42	0,90	0,42	0,30	—
	18	31	24	7	0,49	0,33	0,31	0,35	—
	17	32	34	6	0,66	0,72	—	0,40	0,36
	17	44	30	7	0,76	0,27	0,16	0,16	0,27
	10	59	45	5	0,52	0,58	0,35	0,21	0,31
		Phosphate			0,52	0,31	0,39	0,13	0.27
	10	60	41	5	0,56	0,53	0,34	0,26	0,25
		Phosphate			0,56	0,26	0,29	0,22	0,37
Control Animals	15	28	0		0,62	0,21	0,14	—	—
	15	29	0	8	0,41	0,11	—	0,07	0,14
	17	30	0	7,25	0,35	—	—	0,04	0,07
	24	48	2	4	0,55	0,09	—	—	—
	19	57	1	4	0,63	0,03	—	—	—
	1	69	1	4	0,42	0,01	—	—	—

Commas indicate decimals.

TABLE 2. INFLUENCE OF AMINO ACIDS AND PHOSPHATE ON RN FORMATION

Animal No.	Experiment No.	Reticulocytes %	Conditions	Duration of Incubation, hrs.	RN, mg/ml of Cells: Initial Value	RN, mg/ml of Cells: Increase
1, 24, 24	49, 50, 52	38, 35, 31	NaCl	6	0,67 0,60 0,53	0,24 0,36 0,27
			Phosphate + Glucose		0,67 0,60 0,53	0,03 0,07 0,08
			Phosphate + 11 Amino Acids		0,81 0,70 0,67	0,12 0,15 0,13
			Phosphate + Glucose + 11 Amino Acids		0,81 0,70 0,67	0,08 0,05 0,05
10, 10	61, 66	28, 28	NaCl	5	0,57 0,51	0,32 0,37
			NaCl + Glucose		0,48 0,43	0,20 0,32
			NaCl + 11 Amino Acids		0,59 —	0,02 —
			NaCl + Glucose +11 Amino Acids		0,53 0,56	0,05 0,24
			Phosphate + Glucose		— 0,51	— 0,17
			Phosphate + Glucose + 11 Amino Acids		— 0,57	— 0,18

Commas indicate decimals.

In other experiments the RN formation in the presence of 11 amino acids as as phosphate buffer and glucose was studied, in order to get a better idea of the RN-saving effect. As shown by Table 2, the minimum RN increase was usually observed when glucose and 11 amino acids were added. This is true both of preparations in phosphate buffer and in sodium chloride solution. The saving effect of the glucose seems to be increased in the presence of phosphate buffer. In order to obtain a starting point for the time curve of the RN increase, we determined the dependence of RN increase upon time. Figure 1 shows that the increase in RN decreases slowly with time.

The increase in RN proved to be considerably inhibited by 2,4-dinitrophenol, as shown by Table 3.

TABLE 3. INFLUENCE OF 2,4-DNP ON RN FORMATION

Animal No.	Experiment No.	Reticulocytes %	Incubation period, hrs.	Initial Value	RN Increase Without 10^{-4}-m. 2.4-DNP per ml Cells	RN Increase With 10^{-4}-m. 2.4-DNP per ml Cells
6	26	34	7,5	0,42	0,40	0,24
26	27	46	7	0,53	0,67	0,19
11	42	21	6	0,75	0,33	−0,04
17	44	30	6	0,76	0,27	0,0
8	74	15	4	0,34	0,37	0,14
8	77	36	4	0,50	0,46	0,26
8	83	28	4	0,48	0,31	−0,02

Commas indicate decimals.

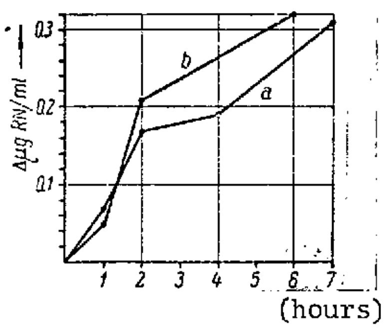

Figure 1. RN Formation Versus Time. Washed blood corpuscles from blood rich in reticulocytes: a) 29.8, b) 15.9% reticulocytes in isotonic NaCl solution at 38°.

2. RN Increase and Hemoglobin Synthesis

From the results of the studies of the behavior of RN, it appeared probable that there is a close relationship between the RN and hemoglobin synthesis. In order to check this assumption, we compared the increase in RN with hemoglobin synthesis under different conditions.

Corresponding experiments are summarized in Table 4. The average values with their mean errors for hemoglobin formation in 7 experiments in NaCl alone amounted to 0.3 ± 0.2%, in 4 experiments in NaCl and glucose 1.6 ± 0.85%, and in 4 other experiments in which glucose and amino acids were added to NaCl, 2.9 ± 0.95% and in 7 experiments in which phosphate (with glucose alone or with glucose and amino acids) was added, 3.4 ± 0.55%. Hence, only the additions in the presence of phosphate or glucose and amino acids are statistically reliable. To some degree, there are reciprocal relationships between the RN increase and hemoglobin formation. In the experiments with NaCl alone, the RN increase was 0.36 ± 0.05 mg, with the addition of glucose 0.29 ± 0.04 and in the presence of amino acids or phosphate, 0.20 ± 0.06 mg/ml of cells.

TABLE 4. RN AND Hb SYNTHESIS

Animal no.	Experiment no.	Reticulocytes	Conditions	Incubation time, hrs.	RN mg/ml of cells: initial value	RN mg/ml of cells: increase	Hb Increase %
24, 24	50,52	35,31	NaCl	6	0,60 0,53	0,36 0,27	0, 0
			Phosphate + Glucose + 11 Amino acids		0,70 0,57	0,05 0,05	+2,9 +3,9
28, 10, 10 10, 10, 10	56, 58, 59 60, 65, 67	12, 43, 45 41, 30, 32	NaCl	5	0,53 0,62 0,51 0,56 0,46 –	0,21 0,26 0,58 0,53 0,33 –	+0,7 +0,8 +0,4 +0,0 +0,3 –
			NaCl + Glucose		0,46 0,62 0,53 0,54 – –	0,19 0,28 0,35 0,34 – –	+3,9 +0,0 +1,7 +0,9 – –
			NaCl + 11 Aminosäuren		0,70 0,56 –	0,13 0,28 –	+1,5 0,0 –
			NaCl + Glucose + 11 Amino acids		0,65 0,73 – – 0,66 0,46	0,07 0,22 – – 0,18 0,34	+5,7 +2,7 – – 2,2 +1,1
			Phosphate		– – 0,57 0,62 – –	– – 0,31 0,26 – –	– – 0,8 +0,9 – –
			Phosphate + Glucose		– – 0,47 0,55 0,37 –	– – 0,39 0,29 0,24 –	– – +6,2 +3,5 +1,9 –
			Phosphate + Glucose + 11 Amino acids		– 0,61 0,57	– 0,15 0,29	– 3,0 +2,5

Commas indicate decimals.

3. RN Increase and Stroma-N /37

From the finding that a significant accumulation of RN takes place during the incubation of blood containing reticulocytes in a medium free of nutrients, the question arises as to where this RN comes from. Corresponding studies are described in Table 5. It follows from the first series of experiments that an increase in RN during incubation in NaCl involves a decrease in stroma-N. Usually, as we can see from the 3 experiments shown, the stroma-N decrease is greater than the RN increase. The next series of experiments shows that 2,4-DNP /38
inhibits stroma-N decrease. The 3 experiments in the last test series show directly the correspondence between the RN-formation and the stroma-N decrease in terms of order of magnitude as well as the inhibition of the transition from stroma-N to residual-N by 2,4-DPN.

TABLE 5. BEHAVIOR OF STROMA-N AND RESIDUAL-N

Animal no.	Reticulocytes, %	Incubation time, hrs.		Initial value	ΔN NaCl	ΔN NaCl + DNP
29	38	4	Stroma-N	3,03	−0,50	
			Res-N	0,82	+0,39	
9	34	4	Stroma-N	3,64	−0,68	
			Res-N	0,68	+0,39	
8	31	4	Stroma-N	2,26	−0,52	
			Res-N	0,60	+0,40	
8	44	6	Stroma-N	2,58	−0,91	−0,12
9	42	4	Stroma-N	4,36	−0,70	+0,03
8	28	4	Stroma-N	2,43	−0,56	−0,08
			Res-N	0,47	+0,32	+0,00
8	31	4	Stroma-N	2,07	−0,75	−0,05
			Res-N	0,36	+0,31	+0,08
29	40	4	Stroma-N	3,19	−0,27	+0,03
			Res-N	0,78	+0,45	+0,08

Commas indicate decimals.

Discussion

After it was proven possible in orientational experiments to exclude the possibility that the RN increase could be attributed solely to the accumulation of the final products of metabolism, it seemed likely that it was an expression of the inhibition of the regeneration of protein with simultaneous protein catabolism, in short, a disturbance of metabolism with resultant catabolic and inhibited anabolic activity.

RN formation is closely related to the state of development of the red blood corpuscles. It is only when the reticulocytes appear in large numbers that one can see a significant increase in RN, while mature erythrocytes exhibit no tendencies toward RN accumulation. However, no direct relationship can be found between the number of reticulocytes and respiration or RN increase. This is also understandable, since the reticulocytes exhibit differences in their metabolic behavior and in hemoglobin content as a function of the severity and duration of anemia [7].

Without a doubt, a number of other factors such as individual differences in the animals, nutrition and functional state of the bone marrow play a role in the considerable variations in the results.

It is worth noting the effect of 2,4-DNP; the inhibition of a process which liberates amino acids by 2,4-DNP was observed by Simpson [8] in liver sections; however, his experimental system, in which an excess of methionine is added to reduce the reincorporation of the liberated amino acids, does not exclude the possibility that the DNP sensitive reaction affects the passage of the amino acids to the cell membrane or their concentration gradient between the cell and the medium. On the contrary, it is clear in our experiments that the inhibition of an intracellular process is involved. Probably there is some relationship between the RN formation, i.e., protein catabolism and oxidative phosphorylation. The fact that the inhibition of RN increase cannot be explained by increased use of the RN for protein synthesis can only be derived from the inhibition of the incorporation of amino acids into a fraction which is insoluble in trichloroacetic acid [9]. This is demonstrated by the experiments described herein, in which 2,4-DNP inhibits the catabolism of the stroma. Possibly the responsible /39 mechanism is the same one as in the system described by Spiegelman and Reiner [10, 11]. They found that the disappearance of induced enzymes in bacteria is related to the presence of an energy source and can be suppressed by "decouplers".

The RN increase is also no doubt closely related to the substrate of the reticulocyte metabolism under different experimental conditions. From this standpoint, the inhibition of RN formation in the presence of glucose can be interpreted as a protein-saving effect, while phosphate, besides having a similar influence, also inhibits respiration. The increasing effect of glucose under anaerobic conditions is perhaps an expression of the utilization of phosphate energy from glycolysis for the catabolism of protein.

As far as we know, the experiments described here are the first in which an increase in hemoglobin was determined *in vitro*. Previous experiments have limited themselves to the detection of the incorporation of labelled amino acids in hemoglobin [9, 12, 13]. The possibility that there could be a clear difference between the net hemoglobin increase and the incorporation of amino acids was discussed by Borsook himself [14] and mentioned by Gale [15] in the pioneering papers on the subject.

The optimum conditions for hemoglobin synthesis require the presence of amino acids, glucose and high concentrations of phosphate. But the presence of glucose and phosphate itself causes increased hemoglobin even without the addition of the amino acids. It still remains to be determined whether phosphate exerts a direct influence or produces its effect indirectly through a reconstruction of the ribonucleic acid fraction [16]. On the whole, it seems that the use of phosphate increases the useful effect of metabolism, and this is particularly clear when hemoglobin synthesis, RN increase and reduced oxygen consumption [4] are compared.

By using various conditions, namely (1) nutrient-free NaCl solution and (2) in the presence of 2,4-DNP, it becomes possible to inhibit selectively the flow of protein conversion to hemoglobin formation, while DNP suppress stroma catabolism and the loss of nutrient or phosphate. These relationships are shown in the following diagram (Figure 2).

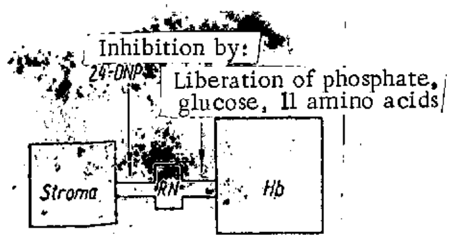

Figure 2. Diagram of the Conversion of the Stroma into Hemoglobin (Hb).

The question arises as to what degree /40
the stroma protein can be viewed as the principal source of hemoglobin synthesis. In this connection, the data are of interest, which indicate that the amount of stroma in the reticulocytes is 2 1/2-4 times the amount in mature erythrocytes [17]. On the basis of

our determinations carried out on the stroma-N, the amount of stroma protein can be estimated as being approximately 2 1/2 times that in normal blood, which means a 5-8 fold amount of stroma for pure reticulocytes as compared with normocytes. In the experiments described here, the decrease in stroma-N and hemoglobin synthesis are on the same order of magnitude. It is therefore clear that the total hemoglobin synthesis in the course of all the stages of the development of the red blood elements in the bone marrow from the stroma-N can in no way be disputed. On the other hand, all the facts seem to indicate that the mature erythrocyte has a higher protein content than any of its previous stages. No doubt the accumulation of amino acids plays an important role in protein formation by red blood cells [18].

Another question involves the homogeneity of the stroma fraction. Even in mature erythrocytes the stroma is considered to be biologically and chemically heterogeneous. In addition to scleroproteins, enzyme proteins such as ATPase [19] and DPN-nucleosidase [20] and proteases [21] can be detected for example. The heterogeneity and the richness in enzymes of the reticulocyte stroma are even pronounced. In particular, the presence of the succinate oxidase system indicates that more highly organized enzyme systems which are mitochondria-like are present [3]. One of the tasks which requires immediate attention therefore is that of characterizing in greater detail the stroma fraction which is the source of the RN formation.

If we consider the processes of protein metabolism from the biological standpoint, the significance of hemoglobin formation in the conversion of protein appears to take place in favor of a highly specialized protein body at the expense of enzyme proteins and subcellular organelles which are associated /41
with the formation of most other cells, especially all of the pluripotent cells. The catabolism of enzyme proteins and organelles is closely related to the loss of their function.

Summary

1. During incubation in nutrient-free medium, there is an increase in residual nitrogen in reticulocytes but not in mature erythrocytes of the rabbit.

2. When glucose and 11 amino acids are added, the increase in residual nitrogen is reduced. At the same time, there is an increase in hemoglobin. An optimum effect is produced by high phosphate concentrations.

3. Residual nitrogen formation can be inhibited by 2,4-dinitrophenol.

4. The source of the residual nitrogen is the reticulocyte stroma.

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